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Presence of Antibodies to Human Papillomavirus Virus-Like Particles (VLPs) in 11–13-Year-Old Schoolgirls

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To allow meaningful approaches to vaccine development, it is important to know the extent of exposure to human papillomavirus (HPV) within the general population, and particularly the age at which the at risk population is infected. The humoral response to human papillomavirus is directed largely to conformationally-dependent epitopes on the whole virion. Virus-like particles (VLPs) of HPV types 1, 2, and 16 were produced using a baculovirus expression system, and were used in the intact state as antigen in an indirect ELISA. Anonymised serum samples from a cohort of Edinburgh schoolgirls were tested for the presence of IgG antibodies directed against the VLPs. The reproducibility of the ELISA was assured by repeated testing of control samples, and by testing all samples in duplicate and, where possible, on several occasions. Of 1,192 tested with the HPV16 VLPs, 90 (7.6%) were classified as clearly positive, and a further 87 (7.3%) were positive but close to the cutoff calculated by comparison with a group of consistently negative sera. Antibodies to HPV2 were detected in 37.5% (407/1,139) and antibodies to HPV 1 in 51.9% (558/1,076) of the schoolgirls. Antibodies to both HPV1 and HPV2 were found frequently, being present in 29.7% (295/993) of samples tested; 40 samples had antibodies to all three types. The significance of these results is discussed. ***J. Med. Virol. 56:210–216, 1998.***

KEY WORDS: HPV; virus-like particles; ELISA

INTRODUCTION

Papillomaviruses induce benign, self-limiting, proliferative lesions on mucocutaneous surfaces. They are exquisitely species- and tissue-specific, and undergo replication only in keratinocytes or other cells with the capacity for squamous maturation. The viruses are not classified serologically but on the basis of DNA sequence homology, and at present more than 80 genotypes, each with a predilection for a cutaneous or mucosal surface, have been cloned from clinical biopsies [De Villiers, 1994]. Those genotypes infecting the genital tract are of major importance, since infection with a subset of these viruses, i.e., human papillomaviruses (HPVs) 16, 18, 31, 33, 35, 45, and other minor types, is the major risk factor for the subsequent development of cancer of the uterine cervix in women [IARC Working Group, 1995]. This association between viral infection and carcinogenesis raises the possibility that prophylactic vaccination against these agents would significantly reduce the incidence of this malignancy. However, before the implementation of such vaccination strategies, the role of the humoral immune response and how and when it occurs in the natural history of HPV infection must be understood.

Serological studies on HPV infection have been hampered until recently by the lack of appropriate antigen targets for serological assays. In vitro systems permissive for virus replication generate little virus, and with the exception of HPV1-induced plantar warts, few clinical lesions contain enough virus particles for practical purposes. Serological assays using HPV1 virions clearly showed that the dominant humoral response is to native conformational epitopes on the surface of the intact particle [Steele and Gallimore, 1990], demonstrating that antigen targets in seroassays must include correctly folded capsid protein. This objective has been met for papillomaviruses for which virions are not available, by the demonstration that expression of the capsid proteins L1 and L2 or L1 alone via eukaryotic expression vectors results in the in vitro self-assembly of empty capsids or virus-like particles (VLPs) [Zhou et

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al., 1991; Kirnbauer et al., 1992]. These VLPs are highly immunogenic, present conformational epitopes, and induce high titres of type-specific neutralising antibodies when injected into rabbits [Christensen et al., 1994]. VLPs have been used as the target antigen in ELISA in several studies [Nonnenmacher et al., 1995; Heim et al., 1995; Carter et al., 1996], and it is generally accepted that VLP-based ELISAs have sufficient sensitivity and specificity to provide an accurate measure of exposure to HPV infection within and between populations [Schiller and Roden, 1996].

That the serum IgG response to papillomavirus infection reflects past exposure to the virus was shown more than 20 years ago for cutaneous lesions [Cubie, 1972], using HPV virions extracted from skin warts. Recent studies using VLP-based ELISAs indicate that specific antibody responses to the L1 protein of genital HPVs are common during and after infection [Heim et al., 1995; Carter et al., 1996; Wideroff et al., 1996]. Prospective studies show that 70–90% of women acquiring HPV16 DNA in the genital tract seroconvert, with a mean time of 8 months elapsing between DNA detection in the genital tract and seroconversion [Carter et al., 1996; Grubert et al., personal communication]. To date, many studies using VLP-based ELISAs have targeted sexually active adults where HPV infection is likely to be common and have linked antibodies to specific virus types with disease status [Dillner et al., 1995; Nonnenmacher et al., 1995; Wang et al., 1997]. However, the variability of the interval between infection and seroconversion indicates that serum antibody responses are not useful for diagnosis of current infection in the individual patient, and that it is more appropriate to use serology as a screening test for evidence of past exposure.

A key issue in the natural history of genital HPV infection, and one of importance for prophylactic vaccination, concerns transmission and whether sexual transmission is the only route for the high-risk HPVs. Determination of the antibody status in prepubertal children would provide information on this issue. To address this, we examined 1,192 serum samples from an anonymised cohort of Edinburgh schoolgirls aged 11–13 for the presence of antibodies to HPV1, -2, and -16 VLPs. Our results indicate that 7% of this schoolgirl cohort have antibodies to HPV16 VLPs, compared to 52% for HPV1 and 38% for HPV2.

MATERIALS AND METHODS

Serum Samples

Test samples. In the Edinburgh schoolgirl rubella vaccination programme, all girls aged 11–13 years were offered prevaccination rubella testing to check the level of natural immunity in the population, with vaccine being given only to those with no rubella-specific antibodies. Because of an excellent administrative system and good support, the uptake was very high (>95%) [Cubie et al., 1985]. After rubella testing, the samples would normally have been discarded, but permission was given by Lothian Health (Edinburgh, Scotland) to retain samples for this study, provided they were anonymised. Although the finger-prick samples were small, it was possible during the 1994–1995 school session to retain samples. The samples were renumbered numerically and stored at –20°C. These samples came from girls attending many different schools and represent a cross section of the normal Edinburgh schoolgirl population.

Control samples. Positive control samples were selected from STD patients attending a Genito-urinary Medicine clinic who appeared to have antibodies to HPV16 synthetic peptides [Wikstrom et al., 1992] (peptides kindly provided by Dr. J. Dillner, Karolinska Institut, Stockholm, Sweden) and from patients with cervical lesions known to contain HPV16. Negative controls for all types were selected by repeated preliminary testing of 1 ml serum aliquots donated to the Regional Clinical Virology Laboratory by the Scottish National Blood Transfusion Service for specific use as controls in serological assays. Positive controls for HPV1 and HPV2 were similarly identified from those giving consistently high optical densities (OD) on repeated testing. Two positive and four negative sera in duplicate were included in every ELISA run.

Construction of Baculovirus Expression Vectors

Preparation of the HPV16 VLPs is described in detail in Zhang et al. [1998]. Briefly, the target gene, L1 or L2, was amplified by PCR, digested at the N and C termini, and ligated with T4 DNA ligase to produce an intermediate transfer vector. The recombinant was cotransfected with baculovirus DNA using the BacPak system (Clontech®, Cambridge BioScience, Cambridge, UK) into Sf21 insect cells, and a primary virus stock was produced after 3–5 days incubation at 270C. Recombinant virus was plaque-purified.

The HPV2a L1 ORF was amplified by PCR from the genome cloned in psp65 (kind gift of Dr. E.M. de Villiers, Deutsches Krebs Forschung Zentrum (DKFZ), Heidelberg, Germany), using the primer pair: 5′ GAT CGA AGA TCT ATG TCT TGT GGC CTA AAC GAC 3′ and 5′ GAT CGC GAA TTC CTA ACG CCT TAC CCG TTT TCG 3′. The 1.6-kb L1 PCR product was digested with *Bgl*II/*Eco*RI, gel-purified with QIAEX II (Qiagen, Crawley, UK), and subcloned into the *Bam*HI site of the pBacPAK8 baculovirus transfer vector (Clontech®). The orientation and integrity of the insert were confirmed by sequencing. The recombinant transfer vector was cotransfected with pBacPAK6 genomic DNA into insect Sf21 cells according to the manufacturer's instructions (Clontech®). In the first round of selection for recombinants, 20–30 virus plaques were picked and screened for HPV2 L1 expression by Western blot analysis, using monoclonal antibody Camvir1 [McLean et al., 1990], which recognises a type-common papillomavirus epitope. Recombinant viruses so identified were subjected to three further rounds of plaque purification, and a master stock of recombinant virus was established for VLP production.

HPV1 L1 VLPs were prepared using an identical pro-

tocol. The L1 gene was cloned out from the HPV 1 genome in pSP65 (kind gift of Dr Jane Sterling, Department of Dermatology, Addenbrookes Hospital, Cambridge, UK).

Production of VLPs for Use as Antigen

Sf21 cells were grown to 80% confluence at 27°C and infected with recombinant baculovirus at a multiplicity of infection (MOI) of 10. Cells were harvested 72 hours later by scraping from the growth surface and were pelleted in a bench centrifuge. The pellet (about 10^8 cells) was resuspended in 15 ml of phosphate-buffered saline (PBS) and homogenised with 50–100 strokes in a Dounce homogeniser (BDH, Butterworth, Leics., UK). Nuclei were separated by centrifugation at 2,000*g* for 10 minutes, and the pellet was resuspended in 4 ml PBS and sonicated (Branson Ultrasonics, Hayes, Middlesex, UK) on ice for 30 seconds. The resulting lysate was layered onto 0.75 ml of a 40% (w/v) sucrose-PBS cushion and centrifuged at 34,000 rpm for 2 hours in a Beckmann SW 55 Ti rotor. The pellet was then resuspended in 2 ml PBS followed by 10 seconds of sonication on ice. Caesium chloride (CsCl)-PBS was then added to a final density of 1.33 g/ml CsCl, and the CsCl-lysate mixture was centrifuged for 16 hours at 45,000 rpm. After centrifugation, two bands were clearly identifiable in the tube, and these were collected separately. The density of the collected fractions was determined with a refractometer (Bellingham-Stanley, Ltd., Fisons, Loughborough, UK). The 1.30 g/ml fraction which contained the VLPs was diluted with PBS, and CsCl was removed by centrifugation. The VLP pellet was then resuspended in PBS and stored at 4°C. Protein content of the preparation was determined by the assay of Bradford [1976], and purity was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Coomassie blue staining. The integrity of the VLP preparation was determined by negative staining and transmission electron microscopy.

ELISA

The ELISA was based on the method of Kirnbauer et al. [1992], with minor modifications. Briefly, the wells of microtitre plates were coated with 50 μl of purified VLPs at 10 μg/ml in PBS, pH 7.2, overnight at 40°C, washed three times with PBS, and blocked with 3% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. The plates were washed and used immediately. Serum samples and controls (50 μl) at a dilution of 1 in 20 in PBS + 1% BSA were added to duplicate wells. After 2 hours of incubation at room temperature, they were washed five times with PBS containing 0.05% Tween-20 and 0.1% BSA. Detection was with anti-human IgG (Fab′ fragment) conjugated to alkaline phosphatase for 1 hour at room temperature, with paranitro phenyl phosphate as substrate for 30 minutes. Colour development was recorded by spectrophotometric measurement at a wavelength of 450 nm.

The mean OD of duplicate samples of three negative samples included in each run plus three standard deviations (SD) was calculated to give a cutoff value (COV). ELISA indices (E.I.) for test samples were calculated by dividing the mean OD of the sample by the COV. The mean OD and SD of replicate samples on five consecutive occasions were calculated and used to provide the baseline for Shewhart charts [Westgard et al., 1981]. In subsequent runs, the negative control ODs were plotted to ensure consistency within and between ELISA runs.

RESULTS

Consistency within and between ELISA runs was shown by repeated testing of the same negative control samples. The mean ODs calculated from five baseline assays had a range of 0.124–0.157. The negative control values obtained in the 14 subsequent ELISAs run on separate days were plotted on Shewhart charts, which showed that the ODs of the controls were generally maintained within the acceptable range (mean ± 2 SD) (Fig. 1). It can be seen that in the tenth run, low absorbances were obtained with all negative controls, with both NC2 and NC4 falling just outside the 2 SD range. ODs in the subsequent run had returned to expected values.

Eleven hundred and ninety-two serum samples were tested using HPV16 VLPs, and 177 (14.9%) gave a positive E.I. (>1.0). Repeat testing was carried out on 243 samples, and 90% gave the same result. The ODs ranged from 0.01–0.65. Many sera showed only weak reactivity, with 87 giving E.I. close to the COV. In all, 90 sera (7.5%) were clearly positive (Table I). These results are discussed below.

With HPV2 VLPs as antigen, 407 out of 1,139 (37.5%) samples were positive, of which 22.1% had E.I. > 1.2. A slightly smaller number of samples (1,076) was tested with HPV1 VLPs, and 558 (51.9%) were positive, with 35% having E.I. > 1.2 (Table I). Of the 993 samples tested with HPV1 and HPV2 VLPs, 295 (29.7%) were positive with both antigens. There were 118 (11.9%) samples which only had HPV2 antibodies, and 287 (28.9%) which had HPV1 antibodies alone. The range and distribution of absorbances for all three antigen types are shown in Figure 2.

Sera with all combinations of seropositivity were noted, with 40.1% of positives showing reactivity to all three VLP types, 28.2% showing reactivity to HPV16 and HPV2 VLPs, 9% to HPV16 and HPV1 VLPs, and only 4.0% showing reactivity only to HPV16 VLPs (Table II).

DISCUSSION

The objective of the present study was to determine the extent, if any, of exposure to HPV16 in a presexually active schoolgirl cohort, so that the level of nonsexual transmission of the high-risk genital HPVs could be assessed. Exposure in this cohort was determined by a serological assay using a HPV L1 VLP-

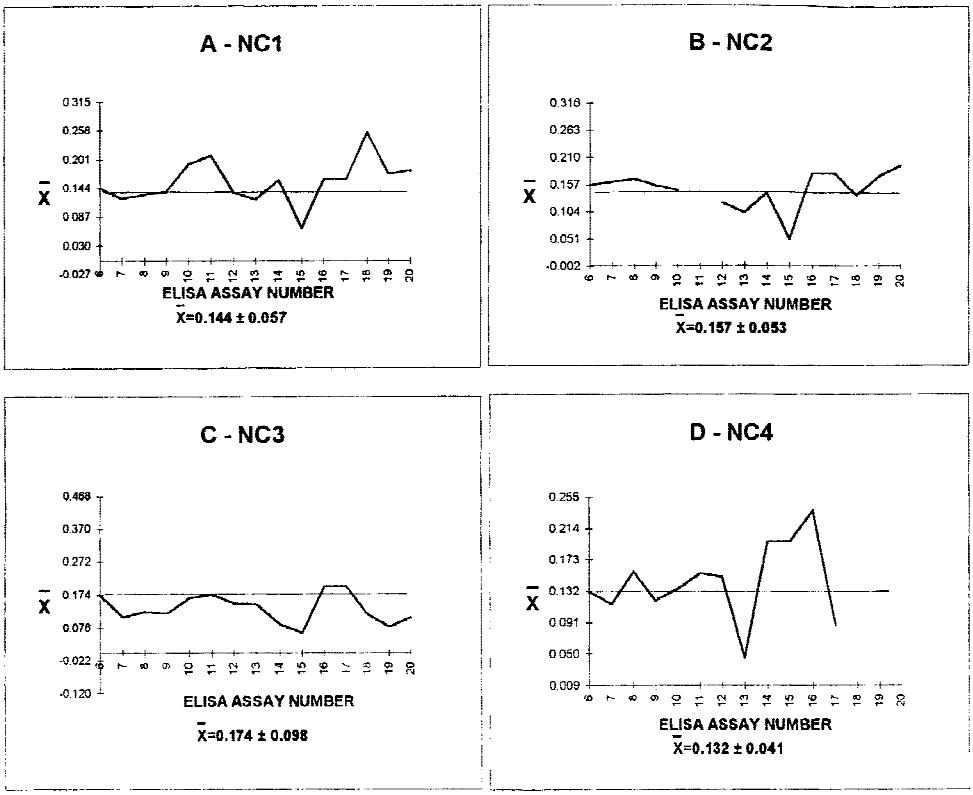

Fig. 1. Shewhart charts of mean Optical Density at a wavelength of 405nm (OD_{405}) readings for four negative control sera (**A–D:** NC1–NC4) obtained in consecutive assays on different days, plotted against the mean and standard deviation values of the five preceding assays.

TABLE I. HPV Seropositivity in Presexually Active Schoolgirls, Using HPV VLPs

HPV VLP type	Number tested	Total number positive (%)	Weak positive/variable (E.I. = 1–1.2) (%)	Positive (E.I. = 1.2–1.5) (%)	Strong positive (E.I. ⩾ 1.5) (%)
HPV16	1,192	177 (14.9)	87 (7.3)	57 (4.8)	33 (2.8)
HPV2	1,139	407 (37.5)	156 (13.7)	117 (10.3)	134 (11.8)
HPV1	1,076	558 (51.9)	184 (17.1)	185 (17.2)	191 (17.8)

based ELISA. Much of the type specificity of the papillomaviruses is attributable to the L1 capsid protein and, indeed, an isolated HPV sequence which shows 90% homology with any known HPV type in the L1 ORF represents a novel type [Van Ranst et al., 1996]. HPV L1 VLPs are identical in size and structure to authentic capsids, and although L2 may contribute to the stability of VLPs [Hagensee et al., 1993], it is not essential for capsid assembly [Kirnbauer et al., 1992]. There is a body of evidence to show that the responses measured in VLP ELISAs are type-specific, and that using VLPs in serological assays gives a good measure of past infection with specific HPV types [Carter and Galloway, 1997].

The schoolgirl cohort whose sera were examined in this study consisted of girls aged 11–13 in the 1994–1995 school intake in Edinburgh, and they represented all social groups in the Edinburgh school system. Our data show that in this cohort, of the 1,192 sera tested with the HPV16 VLP ELISA, 14% gave a positive E.I > 1.0 and, of this group, 7% had an E.I > 1.2. It is possible that a fraction of this schoolgirl cohort of 11–13-year-olds was sexually active and had been so for the interval of 8–12 months after infection, which appears to be necessary for seroconversion. However, it is highly unlikely that the majority of this cohort had been sexually active, and our results support an alternative route of infection.

Vertical transmission has been well-recognised for HPV6/11 in association with juvenile laryngeal papillomatosis, but nonsexual transmission of high-risk genital viruses remains controversial. In a study of virgin teenage girls [Andersson-Ellstrom et al., 1996], none were seropositive in an HPV16 VLP-based ELISA; nor, using a similar assay, were a group of children aged 1–10 years [Schiller and Roden, 1996]. However Rose et al. [1994] found that 2 of 30 children (6%) had anti-HPV16 antibodies, and in a larger study of 155 children aged 1–12 years, Marais et al. [1997] showed 4.5% to be seropositive. There is good evidence

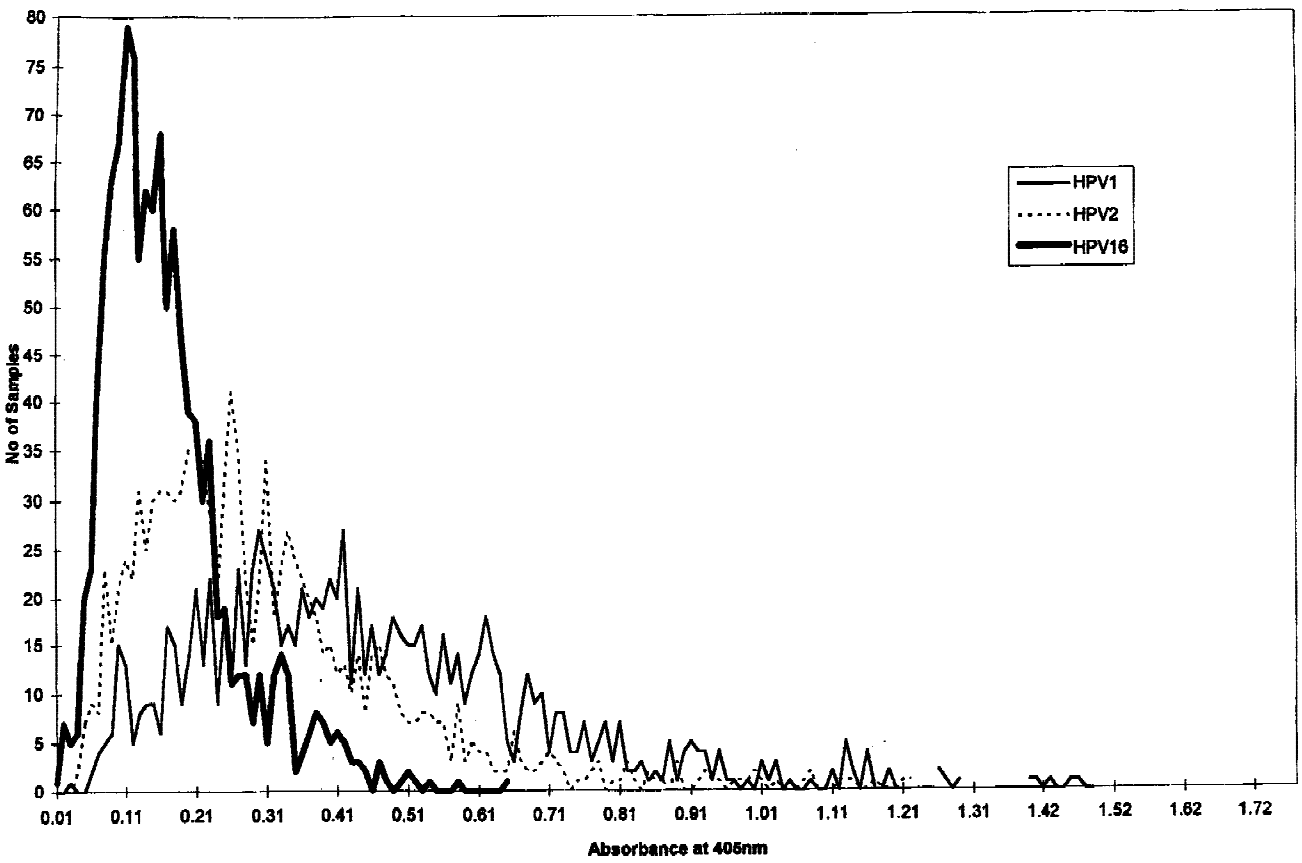

Fig. 2. Range and distribution of absorbances obtained with 1,192 serum samples from schoolgirls when tested by ELISA, using HPV VLP types 1 (——), 2 (------), and 16 (▬).

TABLE II. Detection of Antibodies to Multiple HPV Types by VLP-Based ELISA in 177 Presexually Active Schoolgirls Showing Seroreactivity With HPV16 VLPs

HPV seroreactivity shown with particular HPV VLPs	Number of samples	Percentage of samples
HPV16 only	7	4.0
HPV16 + HPV1	16	9.0
HPV16 + HPV2	50	28.2
HPV16 + HPV1 + HPV2	71	40.1
Not tested for all three types	33	18.6
Total	177	100

for infection with the high-risk HPVs in childhood [reviewed in Cason et al., 1996]. Maternal transmission of HPV16 and HPV18 has been demonstrated unequivocally in about 50% of cases where the mother has these infections at the time of delivery [Sedlacek et al., 1989; Fredericks et al., 1993; Pakarian et al., 1994; Puranen et al., 1997; Tseng et al., 1998], with viral load acting as a major determinant for transmission [Kaye et al., 1994]. Furthermore, recent studies have provided good evidence that vertical transmission of HPV16 can result in long-term persistent infection [Cason et al., 1995] and viral replication in the buccal mucosa [Puranen et al., 1996]. The data obtained in the current study support such an alternative route of exposure.

One of the problems in analysing the data obtained is in defining a truly seronegative population. If one assumes only sexual transmission is possible, then negative controls could be found by using serum from virgins. However, if nonsexual transmission has occurred, some virgins might have specific antibodies, and this would raise the cutoff and reduce the sensitivity of the test. It is clear that during episodes of infection, antibodies develop slowly but are maintained at higher levels than in people who do not have current infections [Carter et al., 1996], that higher absorbances are found in those with persistent or recurrent infections [Widerooff et al., 1996], and that antibody titres can fall rapidly as viral load decreases [Nonnenmacher et al., 1995]. This may simply be because a continuing antigenic stimulus maintains and indeed boosts antibody responsiveness, as seen in many other virus infections.

Our own results show higher absorbances in HPV infections where the exposure to viral antigen is greatest. This is especially true for HPV1, where lesions contain a high viral load of mature particles and exposure to the immune system is consequently greater. This is reflected in our data, in the higher number of girls with strongly positive reactions when HPV1 VLPs were used. HPV2 infections are common in children but they are much more persistent and problematic in adults, possibly because the antibody has waned more

quickly. Certainly a lower percentage of girls were HPV2-seropositive and the strength of the reactions was lower. HPV16 reactivities were even lower, both qualitatively and quantitatively (Table I and Fig. 2).

A key issue arising from this study is the consequence of infection in childhood for the development of cervical disease in adult life, after the individual is reexposed to challenge with the high-risk genital viruses in the genital tract with the onset of sexual activity. Cell-mediated immunity of the Th1 type appears to be essential for viral clearance and the regression of lesions both in humans [Coleman et al., 1994] and in animals [Selvakumar et al., 1995]. In experimental and natural infections in the dog, humoral responses to the capsid proteins of the canine oral papillomavirus accompany the induction of successful cell-mediated immunity and lesion regression (Ghim et al., personal communication). These serum IgG-neutralising antibodies protect against subsequent viral challenge [Suzich et al., 1995]. The situation in genital tract infections in humans is not so clear-cut. In a recent prospective study, IgG responses to HPV16 VLPs were determined in a nonintervention cohort of women with cervical intraepithelial neoplasia (CIN). Specific responses were present in the majority of patients with persistent HPV infection and high-grade CIN, but only in a smaller subset of patients with cleared HPV infection or low-grade lesions [De Gruijl et al., 1997]. However, the isotype of the antibody was not determined in this study, and it is not clear whether these differences reflect the production of neutralising antibody in those who cleared infection, as opposed to nonprotective responses in the persistently infected group. With respect to the responders in the schoolgirl cohort, it is possible that low levels of high-affinity neutralising antibody to the high-risk HPVs would confer protection against reinfection via the genital route. However, the isotype of the antibodies detected in our cohort and their affinity for antigen have not been analysed to date. Until these data are available, it is not possible to speculate on whether the antibody response detected by us represents protective immunity or a nonprotective Th2-type response which could inhibit or suppress effective cell-mediated Th1-based responses in a subsequent infection.

These are important issues to address, particularly with respect to programmes for prophylactic vaccination predicated on the basis that the only route of infection for the high-risk genital viruses is via the genital mucosa. Our data indicate that 7–14% of prepubertal girls have been exposed to HPV16, and this may be an underestimate since in virtually all studies published so far, at least 25% of individuals with detectable HPV DNA in the genital tract were seronegative in the corresponding VLP ELISA [reviewed in Stanley, 1997]. These observations raise the questions of the age at which prophylactic vaccines should be administered and whether seropositive individuals should be identified before mass vaccination.

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